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Molecular Detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in Soil

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PCR primers specific for the *Mycobacterium tuberculosis* complex were used to detect the presence of *Mycobacterium bovis* BCG (Pasteur) in soil microcosms and *Mycobacterium bovis* in environmental samples taken from a farm in Ireland with a history of bovine tuberculosis. *M. bovis* genes were detected in soil at 4 and 21 months after possible contamination. Gene levels were found in the range of 1×10^3 to 3.6×10^3 gene copies g of soil^{-1}, depending on the sampling area. Areas around badger setts had the highest levels of detectable genes and were shown to have the highest levels of gene persistence. *M. bovis*-specific 16S rRNA sequences were detected, providing evidence of the presence of viable cells in Irish soils. Studies of DNA turnover in soil microcosms proved that dead cells of *M. bovis* BCG did not persist beyond 10 days. Further microcosm experiments revealed that *M.* bovis BCG survival was optimal at 37°C with moist soil (−20 kPa; 30% [vol/wt]). This study provides clear evidence that *M. bovis* can persist in the farm environment outside of its hosts and that climatic factors influence survival rates.

Environmental and wildlife reservoirs of *Mycobacterium bovis* are of significance due to the increasing number of bovine tuberculosis breakdowns in cattle herds in both the United Kingdom and Ireland. In Europe, the badger (*Meles meles*) has been implicated, but it is unclear how the disease is transmitted to cattle from badgers. Few studies have considered dissemination and persistence of environmental *M. bovis*. Tanner and Michel (12) reported survival of up to 6 weeks for *M. bovis* cells inoculated into soil and feces, detected by traditional selective cultivation methods. However, cultivation techniques for monitoring *M. bovis* and other mycobacteria in soil are impeded by the slow growth rates of *M. bovis* and the need for prolonged incubation of highly selective agars. Pretreatment or decontamination of samples is required involving the addition of 1 to 5% NaOH, often followed by further treatments with H_2SO_4 (6), oxalic acid (7), or quaternary ammonium compounds (3). *M. bovis* cells surviving for long periods in soil may be sensitive to such harsh pretreatments. No studies have been done to investigate the impact of environmental conditions on the sensitivity of environmental *M. bovis* or the vaccine strain *Mycobacterium bovis* BCG (Pasteur) to these treatments. Methods for the molecular detection of bacterial pathogens in soil have been successfully applied to monitor the fate of salmonellae (10), *Escherichia coli* O157:H7 (5), and *Mycoplasma* sp. (9) by either quantitative PCR (Q-PCR) or reverse transcription-PCR (RT-PCR), thus avoiding the problems of selective cultivation. PCR detection of the *Mycobacterium tuberculosis* complex in clinical specimens has been achieved by targeting antigen genes, such as *mpb70* and *mpb64* (4), and the insertion sequence IS*6110* (1). *mpb70* provides a highly specific and quantifiable target for molecular detection, as it is a single-copy gene found only in members of the tuberculosis complex.

In this study, we present the first report of the use of analysis of community DNA with specific PCR primers targeting both antigen genes and the *M. bovis* 16S rRNA gene to demonstrate the long-term survival of *M. bovis* in environmental samples.

MATERIALS AND METHODS

Bacterial strains. Bacterial species used and culture methods are listed in Table 1. Cultures were incubated at either 30 or 37°C for between 6 and 8 weeks under level 2 containment conditions, with *M. bovis* and *M. tuberculosis* cultured under containment level 3 conditions. Only slow-growing mycobacteria were incubated at 37°C. Cultures were stored in 70% glycerol at −20°C until required.

Farm history and source of environmental samples. Samples were taken from a farm with a history of bovine tuberculosis located in County Louth, Ireland (Ordinance Survey Ireland reference no. O1089). The farm had undergone a tuberculosis infection of cattle 4 months prior to the April 2000 sampling. Following the confirmation of the infection, all cattle and badgers were removed from the farm, with subsequent continuous monitoring for the presence of badger activity. The farm was restocked in January 2001, and a second tuberculosis outbreak was declared soon after, although no badger activity was reported; all cattle were again removed.

In April 2000, 11 plots of 1-m^2 sampling sites were chosen on the farm, ranging from entrances to badger tunnel networks (setts), pastureland on which the infected cattle grazed, and adjoining fields. These sites were designated BS1 and BS2 (badger sett soil), A1 to A3 (pasture soil), and A4 to A9 (remaining sampling sites). Ten cores, each 10 cm in length, were taken from each site. Sampling was repeated in November 2002. As a comparison, soil was also taken from Cryfield Farm, Warwick University, Warwick, United Kingdom (13). This site had no history of use by cattle in the past 20 years. Characteristics of the soils used are given in Table 2.

Nucleic acid extraction from soil. DNA was extracted with the Mobio Soil Microbial DNA Extraction kit per the manufacturer's instructions (Mobio Laboratories, Inc., Solana Beach, Calif.). RNA was extracted from soil with the Mobio Microbial RNA Extraction kit by a slightly modified method. The contents of one extraction tube were added to 1 g of soil in a sterile microcentrifuge tube, and the remaining extraction then continued per the manufacturer's instructions. RNA samples were treated with RNase-free DNase (QIAGEN, Ltd., Crawley, United Kingdom). Purified RNA was checked for the presence of DNA by carrying out a PCR without the RT-PCR step; any samples producing a positive PCR product were retreated with RNase-free DNase, and PCR was carried out again.

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TABLE 1. Bacterial strains used in this study

Bacterial species	Strain no. or source[a]	Growth medium (temp)[b]
Actinomadura malachitica	DSM 43462	1
Actinoplanes auranticolor	DSM 43031	1
Actinosynnema mirum	JCM 3225	1
Pseudonocardia petroleophila	ATCC 15776	4
Arthrobacter oxydans	JCM 2521	1
Brevibacterium flavum	JCM 2180	1
Micromonospora echinospora	ATCC 15838	2
Micromonospora rosea	ATCC 21946	2
Mycobacterium abscessus	JM	3
Mycobacterium agri	JM	3
Mycobacterium aichiense	JM	3
Mycobacterium aurum	ATCC 23366	3
Mycobacterium bovis[c]	Clinical isolate	3 (37°C)
Mycobacterium bovis BCG[c]	Pasteur	3 (37°C)
Mycobacterium chitae	JM	3
Mycobacterium chubuense	JM	3
Mycobacterium duvalii	JM	3
Mycobacterium fortuitum	ATCC 14468	3
Mycobacterium fortuitum	Soil isolate	3
Mycobacterium gadium	JM	3
Mycobacterium genavense	JM	3
Mycobacterium gilvum	JM	3
Mycobacterium gordonae[c]	ATCC 14470	3 (37°C)
Mycobacterium marinum[c]	JM	3 (37°C)
Mycobacterium neoaurum	JM	3
Mycobacterium nonchromogenicum[c]	ATCC 19530	3 (37°C)
Mycobacterium obuense	JM	3
Mycobacterium parafortuitum	JM	3
Mycobacterium peregrinum	JM	3
Mycobacterium phlei	ATCC 354	3
Mycobacterium senegalense	JM	3
Mycobacterium smegmatis	ATCC 13578	3
Mycobacterium terrae[c]	ATCC 15753	3 (37°C)
Mycobacterium thermoresistibile[c]	JM	3 (37°C)
Mycobacterium tuberculosis[c]	Veterinary isolate	3 (37°C)
Nocardia brevicatena	ATCC 15333	4
Planobispora rosea	JCM 3166	1
Planomonospora venezuelensis	ATCC 23865	1
Rhodococcus coprophilus	ATCC 184	1
Rhodococcus luteus	ATCC 35014	1
Streptomyces coelicolor	A3(2)	1
Streptomyces lividans	ATCC 19844	1
Streptomyces violaceus	ATCC 15888	1
Streptoverticillium griseocarneum	ATCC 23934	1

[a] Source of cultures: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama, Japan; ATCC, American Type Culture Collection, Manassas, Va; JM, supplied by John Magee, Regional Centre for Mycobacteriology, Newcastle, United Kingdom.

[b] Growth media: 1, GYM *Streptomyces* medium; 2, *Micromonospora megalomicea* medium; 3, Middlebrook 7H9 broth, Middlebrook 7H10 agar; 4, Glycerol-Sol medium. All media were incubated at 30°C unless otherwise stated (in parentheses).

[c] These species gave PCR products with primer set JSY16SslowF/R.

TABLE 2. Characteristics of soils used

Soil type	Soil composition (% dry wt)	pH (fresh soil)
Warwick	63.6% sand 18.4% silt 11.7% clay Loss on ignition, 6.2%	6.5–6.8
Irish	6.3% sand 22.1% silt 46.4% clay Loss on ignition, 25.1%	7.5–7.7

PCR and RT-PCR. The PCR primers used in this study are shown in Table 3. MPB70F/R, targeting *mpb70*, and MPB64F/R, targeting *mpb64*, were previously used by Gormley et al. (4); the JSY16SslowF/R pair was designed for this project. Alignments were created from known mycobacterial 16S rRNA genes obtained from GenBank. JSY16SslowF was designed to target the long helix insertion (or deletion) present at bases 451 to 482. This is present only in slow-growing mycobacteria. JSY16SslowR was designed to target the *Mycobacterium* genus. These primers were checked for specificity with the species listed in Table 1, and PCR products were obtained only from slow-growing mycobacteria, as indicated in Table 1. PCR with all primer sets was done with the following reaction mixture: $MgCl_2$ (50 μM), Invitrogen PCR buffer (4.5 μl), bovine serum albumin (100 μg ml^{-1}), deoxynucleoside triphosphates (100 μM), primers (0.1 μg of both forward and reverse ml^{-1}), DNA (1 μl), and *Taq* DNA polymerase (Invitrogen, Ltd., Paisley, United Kingdom) (0.3 μl). Reaction mixtures were made up to 46.3 μl with sterile distilled water. PCR was carried out with the following programs. For the MPB64 and MPB70 primers, PCR was at 95°C for 3 min; this was followed by 30 cycles, each consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s; finally, there was a single extension step of 72°C for 5 min. For the JSY16SF/R primers, PCR was at 94°C for 5 min; this was followed by 35 cycles, each consisting of 94°C for 1 min, 55°C for 1 min, and 65°C for 1 min; finally, there was a single extension step of 65°C for 5 min. For the JSY16SslowF/R primers, PCR was at 94°C for 5 min; this was followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, and 67°C for 1 min; finally, there was a single extension step of 67°C for 5 min. RT-PCR was carried out using the reverse transcriptase Superscript II (Invitrogen, Ltd.) per the manufacturer's instructions.

Semiquantitation of PCR products. To determine when the PCR remained linear for all primer sets, a PCR was carried out using 35 replicates. One replicate was removed after each PCR cycle and immediately stored at −20°C. These replicates were then visualized on a 1% agarose gel, and images of the DNA fragments were taken by a UVI tech gel documentation system and saved in an uncompressed TIFF format. The images were then analyzed for pixel intensity of each band with the TotalLab 1d gel program (Nonlinear Dynamics, Ltd., Newcastle, United Kingdom). A set of standards was created using a dilution series of *M. bovis* BCG (Pasteur) with 10^9 to 10^1 cells ml^{-1}. Each dilution was inoculated into 1 g of sterile Warwick soil, and the soil was then air dried to create standardized weights and volumes across the experiment and to ensure that the cells were present in soil and not in an aqueous phase of a soil solution. The PCR was carried out, and a calibration curve was created with the TotalLab 1d gel analysis program to determine the pixel intensity of each band. These DNA standards were used in every PCR experiment and were run alongside unknown products. PCR analysis was carried out on Warwick and Irish soil community DNA with primer sets MPB64F/R and MPB70F/R, with only a 28-cycle reaction used. PCR was also carried out on the *M. bovis* BCG DNA dilution standards at the same time. All products were visualized on the same agarose gel and quantitated using the TotalLab 1d gel analysis program to measure the pixel intensity of bands.

Cloning and sequencing of PCR products. PCR products were ligated into TA cloning vectors (Invitrogen) per the manufacturer's instructions. Chemically competent TOPO *E. coli* cells (Invitrogen) were transformed with the resulting plasmids and subsequently plated out onto Luria-Bertani agar plates containing 100 μg of ampicillin/ml, X-Gal (5-bromo-4-chloro-3-indolyl-ā-D-galactopyranoside), and IPTG (isopropyl-ā-D-thiogalactopyranoside). Colonies were picked for plasmid sequencing with blue-white selection. For extraction of plasmid DNA, transformed *E. coli* colonies were cultured in Luria-Bertani broth containing 100 μg of ampicillin/ml overnight at 37°C. Plasmid DNA was then extracted with QIAGEN Mini-Prep plasmid purification kits per the manufacturer's instructions. For sequencing plasmid DNA, 0.5 μg of template was used with 10 pmol of primer and 4 μl of Big Dye enzyme mixture (PE Applied Biosystems, Foster City, Calif.). The PCR protocol consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products were precipitated by the addition of 1 μl of 3 M sodium acetate (pH 5.2) and 25 μl of ethanol and placed at −20°C for at least 30 min. The samples were centrifuged at 10,000 × *g* for 30 min, the supernatant was discarded, and the pellet was washed with 100 μl of 70% ethanol and allowed to dry. The dried pellet was resuspended in loading buffer and electrophoresed on a polyacrylamide sequencing gel in an ABI 377 sequencer (PE Applied Biosystems).

Microcosm experiments. Warwick soil samples were dried and sieved using a 4-mm mesh; if required for sterile microcosms, the samples were then autoclaved at 121°C for 1 h. This was repeated after 24 h. One gram of either sterile or nonsterile Warwick soil was placed in a sterile universal tube. Microcosms were inoculated with 10^8 *M. bovis* BCG cells, wetted to give a water content of −66 kPa matric potential, and incubated at room temperature unless otherwise stated. Sampling was destructive in all experiments. To monitor cell numbers over time, both plate counts and PCR analysis were carried out for sterile soil

TABLE 3. Primers used to target two antigen genes, *mpb64* and *mpb70,* and 16S rRNA sequences

Primer, sequence	Target gene	Target group of organisms	Amplified product length (bases)	Reference
MPB64F, CAG GCA TCG TCG TCA GCA GC MPB64R, GTG ATT GGC TTG CGA TAG GC	*mpb64*	*M. tuberculosis* complex	543	4
MPB70F, GAA CAA TCC GGA GTT GAC AA MPB70R, AGC ACG CTG TCA ATC ATG TA	*mpb70*	*M. tuberculosis* complex	471	4
JSY16SslowF, CGA CGA AGG TCC GGG TTC TCT CGG ATT GAC JSY16SslowR, GCC ATG CAC CAC CTG CAC ACA GGC CCA C	16S rRNA encoding gene	Slow-growing mycobacteria	605	This paper

microcosms, with PCR analysis used only for nonsterile microcosms. A decontamination method would have had to be used to extract *M. bovis* BCG cells from nonsterile soils; however, our initial studies showed that BCG was either killed or rendered completely unculturable by these methods (15). It was therefore decided to use molecular tools only for nonsterile soil. *M. bovis* BCG cells were extracted from sterile soil by the following method. Ten grams of soil was added to 10 ml of quarter-strength Ringer's solution in a sterile universal tube and shaken for 10 min The soil solutions were mixed, and then 100 μl was plated onto Middlebrook 7H10 (Difco, Franklin Lakes, N.J.) agar plates containing cycloheximide (1 mg ml^{-1}) and nystatin (1 mg ml^{-1}). Plates were incubated in gas-permeable bags for 8 weeks at 30°C. Analysis was carried out at the following time points for all microcosms: 0, 1, 2, 3, 4, 5, 6, 7, 10, 13, 16, 20, 25, 30, and 60 days and then 4, 5, 6, 8, 10, 12, 15, and 18 months after initial inoculation. All experiments were done in triplicate. Separate microcosms were incubated at 4, 15, 25, and 37°C and at 20°C with various water contents of −1,600, −400, −66, −33, −20, and −10 kPa matric potential soil wetting levels. Microcosms were periodically weighed and tested for water loss. Weights were adjusted with sterile water to maintain constant wetting levels.

RNA analysis of microcosms. Duplicate microcosms were set up for all water contents and temperatures in both nonsterile and sterile Warwick soil. Total RNA, including mRNA and 16S rRNA, was extracted from these microcosms at 1, 3, 5, 10, 20, 30, and 60 days after inoculation and from all microcosms at 4, 6, 10, 15, and 18 months with the Mobio Microbial RNA Extraction kit. RT-PCR was carried out using *mpb64*- and *mpb70*-targeted primers. RT-PCR was also done on the total community rRNA present in the extracts with the JSY16SslowF/R primer set. Clone libraries were created, and selected clones underwent plasmid DNA extraction. The 16S cDNA section of the plasmid was then sequenced. As this primer set targets the 16S rRNA genes of all slow-growing mycobacteria, a minimum of 50 clones were sequenced before a microcosm was declared negative for the presence of *M. bovis* BCG 16S rRNA.

Turnover of DNA in soil. Nonsterile Warwick soil microcosms were inoculated with *M. bovis* BCG in the form of live cells, dead but intact cells, and lysed cells and with *M. bovis* BCG DNA. Dead intact cells were created by treating *M. bovis* BCG with UV light for 5 min. Experiments using different lengths of UV exposure showed that 5 min was the optimum time for creating 100% kill without causing lysis. Lysed and intact cells were observed by light microscopy, viability was determined by viable plate counts on Middlebrook 7H10 agar plates, and cells were monitored for growth for a minimum of 10 weeks. Lysed cells were created by incubation of cultures at 100°C for 10 min; again, our experiments showed this to be the optimum temperature and time for maximum cell death and lysis. A total of 10^8 cells (or 10^8 genome equivalents in the case of free DNA) were inoculated into each microcosm. Microcosms were incubated at both 10 and 30°C, with samples taken every day for 21 days. DNA was then reextracted from these samples, and PCR was carried out using *mpb70*- and *mpb64*-targeted primers. Products were visualized, and the pixel intensity of bands was quantified as described above.

Statistical analysis. For microcosm experiments, the decrease day^{-1} rates were calculated as the average decrease in cell numbers (or gene counts) per day. Statistical analysis was done with the Excel program using one-way analysis of variance; *P* values of 0.05 were considered significant.

RESULTS

Primer specificity. The PCR primers listed in Table 3 were used in a PCR on DNA extracted from the bacterial species listed in Table 1. Of all the species tested, only *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG (Pasteur) gave PCR products for both antigen gene primers. The products from all strains were cloned, sequenced, and compared to genomes with the Sanger Institute's *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG (Pasteur) BLAST servers (http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml). PCR products showed 100% similarity to sequences on all three genome assemblies. The JSY16SslowF/R primers were shown to be specific for the slow-growing group of the mycobacteria. No PCR products were detected in any fast-growing species or in any of the nonmycobacterial species tested (as listed in Table 1). Limits of detection for all primer sets were obtained for both PCR and RT-PCR. With PCR, MPB64F/R and MPB70F/R had a lower limit of detection, between 10^1 and 10^2 cells g of $soil^{-1}$, depending on the soil type (Irish soils were at the upper limit), and JSY16SslowF/R had a similar limit of detection. With RT-PCR, MPB64F/R and MPB70F/R had a lower limit of detection of 10^4 cells g of $soil^{-1}$ and JSY16SslowF/R had a lower limit of between 10^2 and 10^3 cells g of $soil^{-1}$.

Effect of temperature and water content on *M. bovis* BCG in soil. In Warwick soil microcosm experiments, cell and gene numbers both decreased over time. Survival rates increased as temperature increased, with lowest survival levels at 4°C (Fig. 1A and B). Analysis of variance was calculated on the temperature data with decrease day^{-1} rates significantly different between 4 and 37°C for all three types of detection (decay rates not shown). Survival was also significantly better in nonsterile soil than in sterile soil at all temperatures. Semi-Q-PCR counts were significantly higher than plate counts at 60 days at all temperatures, except at 37°C for sterile soil microcosms (Fig. 1C).

Survival was optimal at −20 kPa (Fig. 2), with no significant difference between decay day^{-1} rates for plate and gene counts in sterile soil microcosms.

RNA analysis of microcosms. mRNA for both antigen genes was detected in culture filtrates and in total RNA extracted from sterile and nonsterile soil following inoculation at day 0. RNA was extracted from the microcosm soil throughout the 18-month incubation, but no mRNA was detected after day 0. However, 16S rRNA sequences from *M. bovis* could be detected in all microcosms up to the 60-day extraction. The comparison of 16S rRNA detection for various temperature and matric potentials supported the Q-PCR counts, with optimal survivals at 37°C and −20 kPa (Table 4).

Turnover of *M. bovis* BCG DNA in soil. Complete cell death was noted at both the optimal UV exposure times and the optimal heat-killing times. This was determined by viable cell

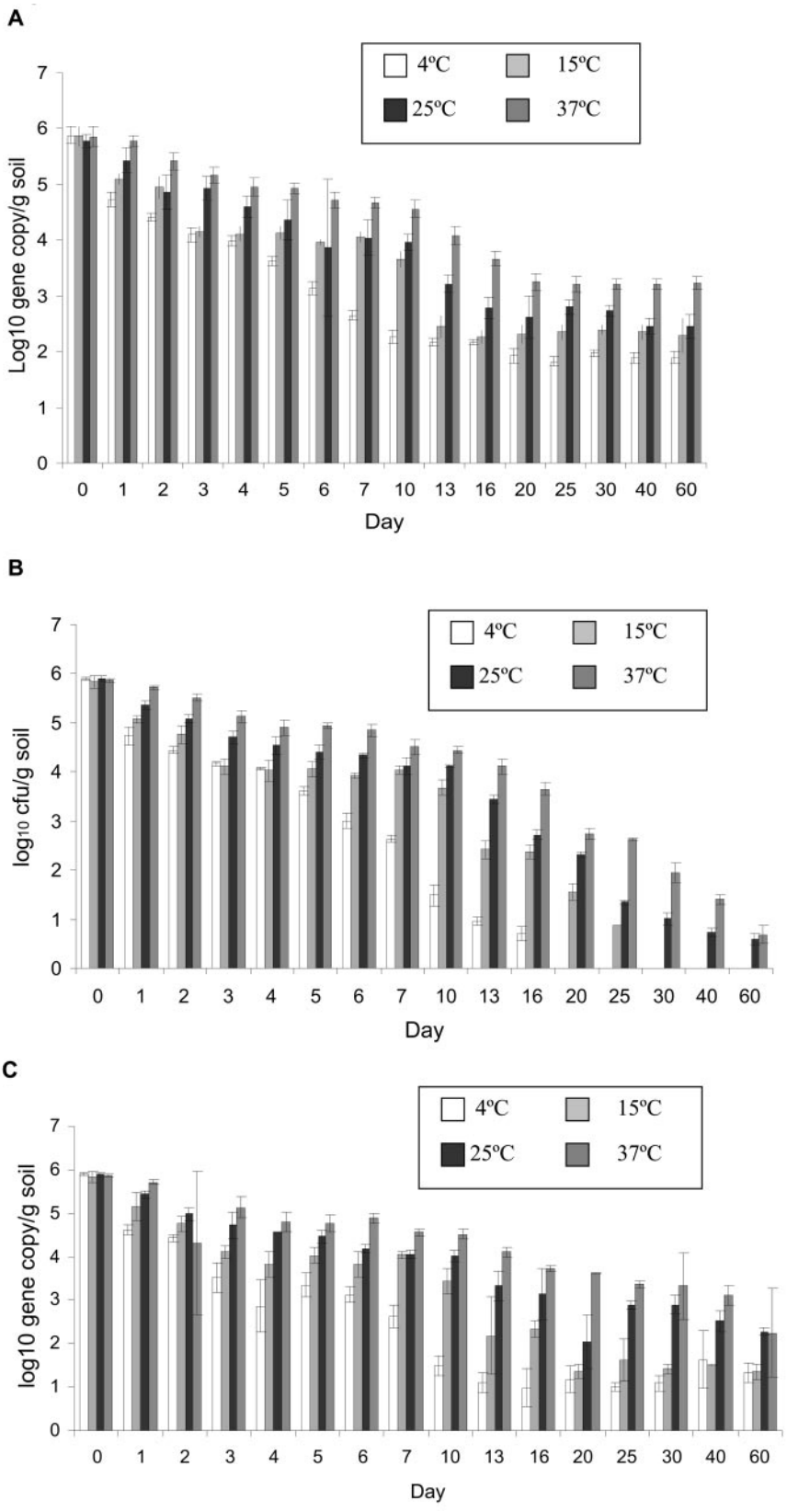

FIG. 1. Detection of *M. bovis* BCG in 1 g of sterile soil microcosms monitored by direct gel quantitation of PCR products (A), 1 g of sterile soil monitored by culturable cell counts (B), and 1 g of nonsterile soil microcosms monitored by direct gel quantitation (C). Error bars represent standard deviation (SD) values.

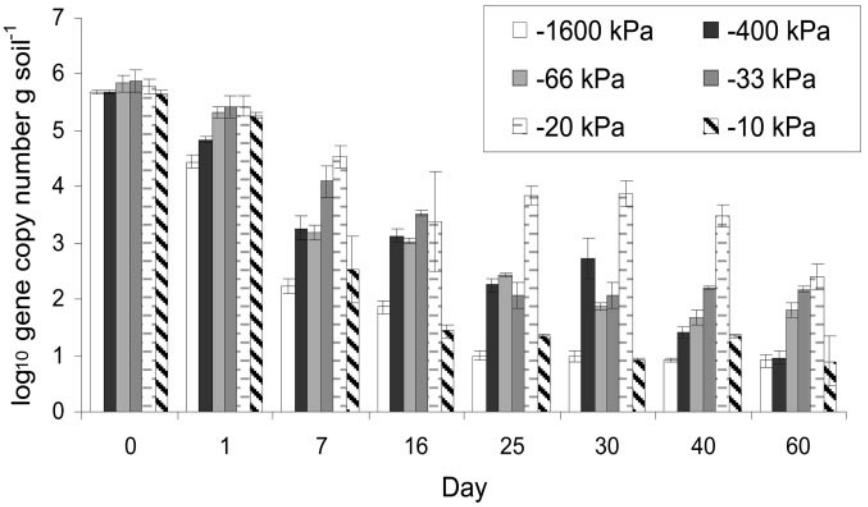

FIG. 2. Comparison of *M. bovis* BCG antigen gene counts in nonsterile soil microcosms under various water contents (in kilopascals). Error bars represent SD values.

counts. DNA turnover in soil was most rapid for cell-free DNA (Fig. 3). A comparison of DNA turnover in dead and live cells proved that DNA in dead cells did not persist beyond 10 days under optimal survival conditions. DNA within live cells persisted for the duration of the experiment (20 days) and was stable at both 10 and 37°C in nonsterile soil (Fig. 4).

Sampling of a farm site in Ireland. Screening of April 2000 soil samples for both *mpb64* and *mpb70* revealed the presence of the target genes in badger setts (BS1 and BS2) and adjacent fields (A1 and A2). No PCR products were recovered from any other soil sample (A3 to A9) (Fig. 5). Semiquantitation of the PCR products indicated levels of 1×10^3 to 3×10^3 gene copies g of soil^{-1} (Table 5). Soil samples taken in November 2002 gave PCR products for BS1 and BS2 cores but not for any other samples, with gene levels of 2.15×10^3 and 2.5×10^3 gene copies g of soil^{-1}, respectively (Table 5). No mRNA for *mpb64* and *mpb70* was detected in any of the soil samples. Confirmation of the presence of *M. bovis* in BS1, BS2, A1, and A2 soil samples was achieved by RT-PCR with the JSY16Sslow primer set (targeted to the 16S rRNA of the slow-growing group of mycobacteria). Sequences with >99% identity to *M. bovis* were found in all soil cores that had given a PCR product for the antigen genes.

TABLE 4. Last month at which 16S rRNA from *M. bovis* BCG could be detected in different soil microcosms

Water content (kPa)	Temp (°C)	Presence of 16S rRNA (mo)	
		Sterile soil	Nonsterile soil
−66	4	6	10
	15	10	10
	25	15	10
	37	15	15
−1,600	22	4	3
−400		4	3
−66		10	10
−33		15	10
−20		15	15
−10		3	3

DISCUSSION

The molecular monitoring of environmental *M. bovis* in this study has clearly shown survival in both monitored farm field sites and controlled soil microcosms. The successful detection of an environmental reservoir of *M. bovis* cells was achieved using a specific PCR, avoiding the problems of attempting cultivation of this slow-growing group of bacteria. We have proved that detection of target DNA is directly correlated to the presence of viable *M. bovis* BCG cells in microcosm studies, as DNA decay rates clearly showed DNA did not persist in dead cells in soil. It is possible that the decay rate for extracellular DNA may vary by soil characteristics. Romanowski et al. (12) studied the persistence of plasmid DNA in soil and observed rapid decline over a period of 10 days; however, soil type did affect the rate of decay. The survival rates for *M. bovis* in sterile soil at 37°C were different for DNA (live cells) and viable cell count estimates, as the counts indicated a much more rapid decay over a 2-week period. This difference may be due to changes in culturability of the cells in soil. The molecular detection of bacterial pathogen DNA in soil clearly demonstrates that the pathogen was present in the soil, but its current physiological state is more difficult to determine. The detection of 16S rRNA sequences identical to those of *M. bovis* in RNA recovered from the Irish soils provides further evidence for the presence of viable cells. There is no published data indicating the persistence of RNA in soil.

The chosen Irish farm site was closely monitored for incoming badgers following the initial culling, but none were detected. Therefore, we attributed the continuing presence of *M. bovis* DNA in setts and adjacent pasture to the survival of environmental *M. bovis*. This survival in soil was clearly supported by the microcosm studies, which revealed that damp, warm soil was optimal for *M. bovis* BCG survival. The BCG

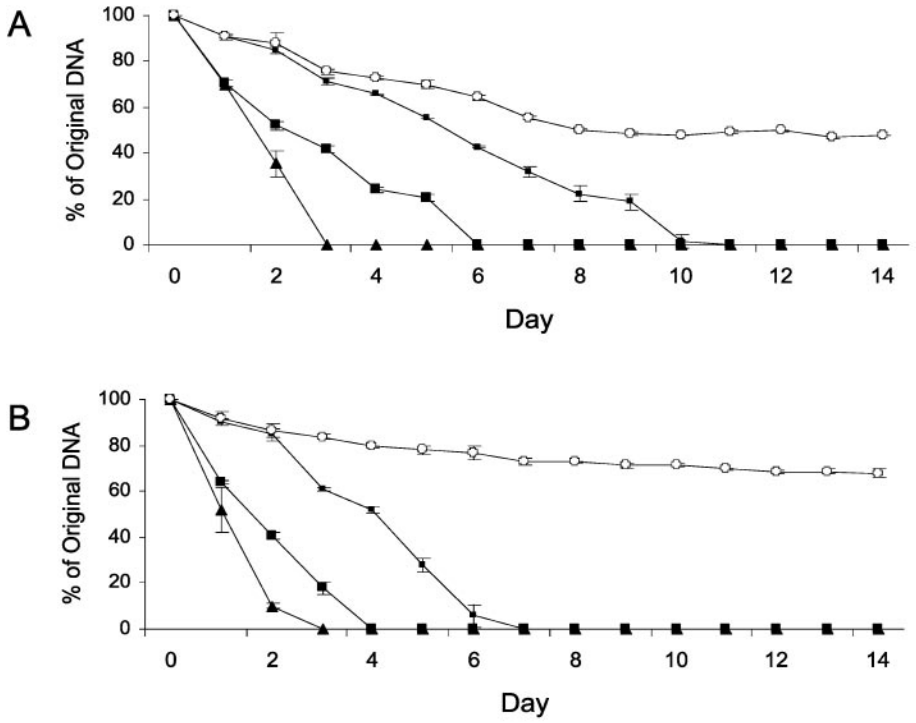

FIG. 3. Persistence of *M. bovis* BCG DNA from four sources in soil microcosms held at 10°C (A) and 37°C (B). ▲, DNA; ■, DNA from dead, lysed cells; ●, DNA from dead intact cells; and ○, DNA from live intact cells. DNA was measured by quantitation of PCR products obtained with primer sets for *mpb64* and *mpb70* in a 30-cycle reaction mixture. Error bars represent SD values.

strain is an attenuated mutant of the *M. bovis* wild type (11) and as such is likely to be more sensitive to environmental conditions. The surprising findings of improved survival in nonsterile soil compared to sterile soil may be related to the activity of the indigenous soil microbial population, possibly by providing cofactors for cell repair and maintenance. The sterile soil used would also contain nutrients and possibly toxins from lysed bacterial biomass in situ, and these nutrients may keep the cells in an active rather than a dormant state. The latter may be readily induced in a natural soil under more oligotrophic conditions, and dormant cells would survive better than acting ones.

There have been a number of studies on the cultivation and characterization of *Mycobacterium* spp. from environmental samples (6, 7), but *M. bovis* strains have never been isolated. Little et al. (8) monitored infected badgers kept in an isolated yard for tuberculosis infection and any subsequent shedding of bacteria. As part of the experiment, environmental samples from the yard, including badger feces, soil, hay, scrapings from feeding bowls, and water, were examined for the presence of *M. bovis*. Despite the animals shedding large numbers of *M. bovis* cells in urine and sputum, *M. bovis* was not isolated at any time. Duffield and Young (2) could not detect *M. bovis* in

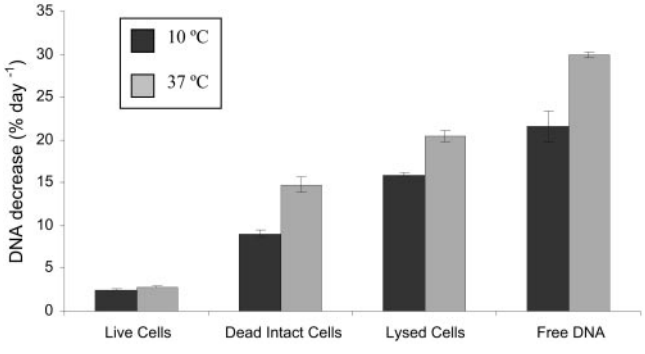

FIG. 4. Rates of decay per day of *M. bovis* BCG DNA from four different sources held at 10 and 37°C. Error bars represent SD values.

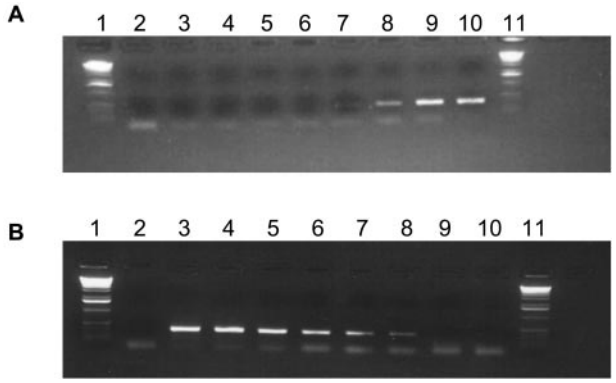

FIG. 5. Detection of antigen gene PCR products. (A) *mpb64.* Lanes: 1, molecular markers; 2, negative control; 3, A6; 4, A5; 5, A4; 6, A3; 7, A2; 8, A1; 9, BS1; 10, *M. bovis* BCG; 11, molecular markers. (B) *mpb70.* Lanes: 1, molecular markers; 2, negative control; 3, *M. bovis* BCG; 4 to 6, BS1; 7, A1; 8, A2; 9, A3; 10, A4; 11, molecular markers.

TABLE 5. Quantitation of PCR products obtained from community DNA extracted from soil

Sampling date (mo yr)	Sample code	Positive soil cores (%)[a]	No. of gene copies g of soil^{-1}[b]
April 2000	BS1	90	3,612 ± 147.24
	BS2	90	2,544 ± 41.47
	A1	90	1,584 ± 36.50
	A2	80	1,003 ± 6.08
	A3–A9	0	Below limits of detection
November 2002	BS1	80	2,528 ± 258.76
	BS2	80	2,151 ± 231.86
	A1–A9	0	Below limits of detection

[a] Percentage of 10 soil cores with triplicate DNA extractions from each core.
[b] Values are means ± standard deviation.

artificially infected soil and feces samples after 4 weeks. It seems likely that the failure to selectively isolate *M. bovis* from soil was due to problems of decontamination of the soil to allow long-term incubation of isolation plates. Our own studies have shown that *M. bovis* cells recovered from soil are highly sensitive to decontamination by standard procedures (14).

The long-term presence of *M. bovis* BCG 16S rRNA sequences recovered following RT-PCR of RNA extracts from sterile soil microcosms proved that although cells became unculturable, they were still present. Further evidence for the presence of intact viable cells in the farm environment was obtained, following detection of *M. bovis*-specific 16S rRNA sequences by RT-PCR. The lack of mRNA for the targeted antigen genes in RNA extracted from soil was not surprising, as these genes are probably not expressed in the soil environment. From the evidence presented here, it can be concluded that *M. bovis* BCG remains viable in soil for more than 15 months and that significant levels of *M. bovis* DNA and RNA persist in the field, indicating the presence of viable cells as an environmental reservoir for infection, which may pose a risk to cattle.

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